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Influence of Plant Polymers on the Distribution and Cultivation of Bacteria in the Phylum *Acidobacteria*▽†

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Members of the phylum *Acidobacteria* are among the most abundant bacteria in soil. Although they have been characterized as versatile heterotrophs, it is unclear if the types and availability of organic resources influence their distribution in soil. The potential for organic resources to select for different acidobacteria was assessed using molecular and cultivation-based approaches with agricultural and managed grassland soils in Michigan. The distribution of acidobacteria varied with the carbon content of soil: the proportion of subdivision 4 sequences was highest in agricultural soils (ca. 41%) that contained less carbon than grassland soils, where the proportions of subdivision 1, 3, 4, and 6 sequences were similar. Either readily oxidizable carbon or plant polymers were used as the sole carbon and energy source to isolate heterotrophic bacteria from these soils. Plant polymers increased the diversity of acidobacteria cultivated but decreased the total number of heterotrophs recovered compared to readily oxidizable carbon. Two phylogenetically novel *Acidobacteria* strains isolated on the plant polymer medium were characterized. Strains KBS 83 (subdivision 1) and KBS 96 (subdivision 3) are moderate acidophiles with pH optima of 5.0 and 6.0, respectively. Both strains grew slowly ($\mu = 0.01\ h^{-1}$) and harbored either 1 (strain KBS 83) or 2 (strain KBS 96) copies of the 16S rRNA encoding gene—a genomic characteristic typical of oligotrophs. Strain KBS 83 is a microaerophile, growing optimally at 8% oxygen. These metabolic characteristics help delineate the niches that acidobacteria occupy in soil and are consistent with their widespread distribution and abundance.

Soil is a major reservoir of organic carbon for the planet (77), estimated to contain ca. 3.3 times more carbon than the atmosphere (33). Plant biomass is an important source of organic carbon in fertile soils. Two broad categories of plant carbon include simple or readily oxidizable carbon (e.g., glucose) arising from root exudates and plant polymers (e.g., cellulose, xylan) derived from the structural components of plants.

Microbial communities in soil are influenced by inputs of these types of plant carbon. Throughout the growing season, plant roots release readily oxidizable carbon into the surrounding soil, which influences the structure of the rhizosphere community (18). Plant polymers are supplied in plant litter primarily after harvest (76). Although tillage will oftentimes lead to the loss of stored soil organic carbon (51), it does distribute plant polymers throughout the upper layers of the soil (2). Soil microorganisms are responsible for the breakdown and cycling of these plant polymers. For example, select members of the *Bacillus*, *Pseudomonas*, *Cytophaga*, *Cellulomonas*, *Actinomycetes* (79), *Streptomyces*, and *Micromonospora* (43) are capable of aerobic cellulose degradation. However, the capacity to break down plant polymers has not been explored in the phylum *Acidobacteria*.

Acidobacteria have been detected in various environments, including soils and sediments (5, 14), soil crusts of sand dunes (67), wastewater (10, 36), water distribution systems (44), peat bogs (12), acid mine drainage (26), hot springs (19), shallow submarine hydrothermal vents (65), the surface of Paleolithic cave paintings and catacombs (59–61, 80, 81), and uranium-contaminated subsurface sediments (4). There are 26 recognized acidobacterial subdivisions but very few isolates (11–13, 15, 17, 22–24, 56–58, 70). Members of this phylum have been difficult to culture and maintain in the laboratory. Our knowledge of their metabolic capabilities and potential role(s) is rudimentary. Data from whole-genome analysis of three strains suggest that at least some acidobacteria are versatile heterotrophs capable of using both plant polymers and readily oxidizable carbon (75).

The ubiquity of acidobacteria across soils that differ in carbon content and the ability of strains to use both readily oxidizable carbon and plant polymers suggest that members of this phylum might be equipped to use various carbon sources. To explore this idea, the ability of acidobacteria to use readily oxidizable carbon and plant polymers was examined with agricultural and managed grassland soils in Michigan using a combination of molecular and cultivation-based methods. The distribution of acidobacterial subdivisions between the agricultural and managed grassland soils in Michigan, the former presumed to be enriched with plant material due to tillage, was characterized to assess subdivision-level differences. Using these soils, total aerobic heterotrophs were isolated using plates amended with either readily oxidizable carbon or plant polymers. These cultivation plates were screened for the presence of acidobacteria, and the 16S rRNA gene sequences derived from plate wash DNA of enrichment plates amended with either readily oxidizable carbon or plant polymers were

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TABLE 1. Soil pH, percent soil moisture, carbon percentages, and nitrogen percentages for field replicates of the agriculture and managed grassland soils

Treatment	Field replicate	pH[a]	Soil moisture (%)[a]	Carbon (%)[b]	Nitrogen (%)[b]
Conventional agriculture, corn/soybean/wheat rotation with chemical input	R1	6.06 ± 0.05	14.94 ± 0.15	0.73 ± 0.17	0.08 ± 0.03
	R2	6.28 ± 0.04	15.49 ± 0.06	0.86 ± 0.24	0.10 ± 0.03
	R4	6.63 ± 0.05	16.02 ± 0.03	0.74 ± 0.14	0.09 ± 0.03
	R5	5.81 ± 0.03	15.20 ± 0.08	0.79 ± 0.17	0.09 ± 0.02
Treatment avg ± SD		6.19 ± 0.35	15.41 ± 0.46	0.78 ± 0.06	0.09 ± 0.01
Managed grassland soil, never plowed	R1	6.06 ± 0.06	23.57 ± 0.15	1.55 ± 0.43	0.14 ± 0.04
	R2	6.19 ± 0.04	23.84 ± 0.10	1.60 ± 0.51	0.14 ± 0.04
	R3	6.12 ± 0.04	25.41 ± 0.70	1.61 ± 0.49	0.15 ± 0.05
	R4	6.41 ± 0.02	23.17 ± 0.25	1.53 ± 0.42	0.15 ± 0.04
Treatment avg ± SD		6.19 ± 0.15	24.00 ± 0.98	1.57 ± 0.04	0.14 ± 0.006

[a] Indicates average ± standard deviation (SD) of results for two technical replicates using standard methods (53).
[b] Carbon and nitrogen percentages are given as averages ± standard deviations of the percent elemental carbon and nitrogen (g C or N per 100 g soil) from data collected between 1989 and 2001 at the KBS LTER (http://lter.kbs.msu.edu/datatables). Values were determined by sample combustion and subsequent TCD gas chromatography (Carlo Erba automated CHN analyzer).

assessed for differences in acidobacterial composition. Two novel acidobacterial strains capable of degrading plant polymers were isolated and selected for further characterization. This study provides insight into the subdivision-level distribution of acidobacteria between agricultural and managed grassland soils in Michigan, the influence of carbon content on that distribution, the effects of readily oxidizable carbon and plant polymers on the cultivation of acidobacteria, and two newly characterized plant polymer-degrading strains.

MATERIALS AND METHODS

Phylogenetic survey and isolation of strains. (i) Soil description. Soil samples were collected from the Michigan State University W. K. Kellogg Biological Station Long-Term Ecological Research (KBS LTER) site (http://www.kbs.msu.edu). The KBS LTER is a 60-hectare research site established in 1989 to study ecological processes in agroecosystems. The site has been subdivided based on a randomized block design into seven 1-hectare management regimes with five randomized sampling sites per 1-hectare replicate plot (54). The dominant soil types are of the Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs) series.

Portions of the soil were used to determine pH and moisture using standard methods (53). The carbon and nitrogen percentages (g C or N per 100 g soil) were determined using TCD gas chromatography (Carlo Erba automated CHN analyzer) collected between 1989 and 2001 at the KBS LTER (http://lter.kbs.msu.edu/datatables). Soil properties are depicted in Table 1.

(ii) DNA-based surveys. DNA-based surveys of the acidobacterial subdivisions were generated from two management regimes: a conventional agriculture site with a rotation of corn/soybean/wheat and a never-tilled successional plant community that is populated primarily by grasses due to annual mowing. Soil cores were collected in July 2005 from four field replicates from the aforementioned management regimes. Briefly, five cores (2.5-cm diameter by 7-cm depth) from each 1-hectare plot were pooled and homogenized using a 2-mm sieve.

DNA was extracted from eight composite soil samples using the UltraClean Fecal DNA MoBio DNA extraction kit (MoBio, Carlsbad, CA). A total of eight acidobacterial 16S rRNA gene libraries (n = 100 clones per library) were created, four field replicates of the agricultural soil and four field replicates of the managed grassland soil. The 16S rRNA genes were amplified using an *Acidobacteria*-targeting forward primer (31F: 5′ GAT CCT GGC TCA GAA TC 3′) (5) and a broadly inclusive bacterial reverse primer (1492R: 5′ GGT TAC CTT GTT ACG ACT T 3′) (34, 35), generating a ca. 1,461-bp product. Three amplification reactions were conducted per DNA sample. Each 25-μl PCR mixture contained 1× PCR buffer, 1 mM $MgCl_2$, 0.03 mM each deoxynucleoside triphosphate (dNTP), 0.2 μM each primer, and 5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Thermal cycling consisted of the following steps: (i) 95°C for 3 min; (ii) 95°C for 30 s, 56°C for 30 s, 72°C for 45 s (repeated 30 times); and (iii) 72°C for 10 min. Genomic DNA purified from *Acidobacterium capsulatum* (ATCC 51196) was used as a positive control. The PCR products were separated by electrophoresis with a 1% agarose gel in 0.5× Tris-borate-EDTA (TBE) and visualized with ethidium bromide. Amplicons were pooled, purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and cloned with the Invitrogen TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). Clones were submitted for bidirectional sequencing using the M13 primers at the LANL JGI Sequencing Facility.

(iii) Isolation experiments. Soil samples were collected from the agriculture and managed grassland soils in July 2006 from the KBS LTER site as described above.

Five cores (2.5-cm diameter by 10-cm depth) from one field replicate per site were collected and homogenized with a 2-mm sieve. Portions of the homogenized soil were used to determine soil moisture content (53). Soil suspensions, which were also used to allow for comparison of previous data (15, 70), were made using a well-established method for soil cultivation experiments as described previously (15, 70). Approximately 30 g of soil were added to 100 ml of a phosphate-buffered salt solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4); the solution was adjusted to a pH of 7.0 and supplemented with 2.24 mM $Na_4P_2O_7 \cdot 10H_2O$ as a dispersal agent and 1 mM dithiothreitol as a reducing agent (73). The soil suspension was stirred vigorously with a magnetic stir bar for 30 min on the bench top. Soil aggregates were allowed to settle for 30 min, after which an aliquot of the soil suspension was used to prepare 10-fold decimal dilutions in the same buffer for subsequent inoculation of the isolation medium.

The isolation medium contained (per liter) 1.95 g 2-(*N*-morpholino)ethanesulfonic acid (MOPS), 0.4 mM $MgSO_4$, 0.6 mM $CaCl_2$, 0.4 mM $(NH_4)_2HPO_4$, 2 ml selenite/tungstate solution (68), and 2 ml of SL-10 (78) as described previously as VL (24). Gellan gum (ca. 14 g/liter) was the solidifying agent (24). This isolation medium was amended with one of the following carbon mixtures, prepared in distilled water: (i) a mixture of plant polymeric carbon, including xylan (derived from oat spelts, birchwood, and larch), xanthan, pectin, and methyl cellulose each at 0.05 g/liter; (ii) a mixture of readily oxidizable carbon sources, including yeast extract, Bacto protease peptone no. 3, Casamino Acids, and dextrose at 0.05 g/liter; or (iii) xylan from birchwood at 0.05 g/liter. All carbon compounds were purchased from the Sigma-Aldrich Co., St. Louis, MO. Plates amended with these mixtures were prepared at either pH 5.5 or 6.5. Here, this composition will be referred to as VL-#-(PP, ROC, and xylan). The # indicates the pH, and the carbon mixtures are designated PP (mixture of plant polymers), ROC (readily oxidizable carbon), and xylan.

Plates were incubated for 30 days at 23°C under one of the following atmospheres: CO_2-enriched hypoxia (2% O_2, 5% CO_2, and balance N_2, by volume) or air. The CO_2-enriched hypoxia atmosphere was maintained within a flexible vinyl chamber fitted with an oxygen sensor/controller (Coy Laboratory Products, Grass Lake, MI).

A total of 288 plates containing treatments of soil type, atmosphere, and medium amendments were screened for the presence of acidobacteria after ca.

30 days of growth using plate wash PCR (70) with *Acidobacteria*-targeting primers. Plate wash DNAs that yielded amplification with *Acidobacteria*-targeting primers were purified using ExoSAP-IT (USB, Cleveland, OH) and submitted for sequencing with primer 531R (5′ TAC CGC GGC TGC TGG CAC 3′) at the Michigan State University Research Technology Support Facility (http://rtsf.msu.edu/).

Characterization of two novel acidobacterial strains. (i) Standard growth medium. After initial isolation, acidobacterial strains KBS 83 and KBS 96 were found to grow faster and to a higher density on a modified *Hyphomicrobium* medium 337 (49) (MHM). MHM contained the following (per liter): NaCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; NH_4Cl, 0.25 g; KH_2PO_4, 0.2 g; KCl, 0.5 g; Na_2SO_4, 0.28 g; KNO_3, 5 g; K_2HPO_4, 0.006 g; and KH_2PO_4, 0.019 g; it also contained 20 mM respective buffer, 10 mM designated carbon source, along with 1 ml SL-10 trace element solution, 1 ml vitamin B_{12} (50 mg/ml), and 1 ml vitamin mix as described previously (15). The carbon source was 10 mM glucose for strain KBS 83 or a mixture of organic compounds (yeast extract, protease peptone, Casamino Acids, and glucose, 0.15 g/liter each, referred to as 0.3× R2B) for strain KBS 96.

(ii) Optimization of growth rate. To determine the effect of pH on growth rate, strains KBS 83 and KBS 96 were grown at room temperature (23°C) between pH 3.0 and 7.0 at intervals of 0.5 pH units. The MHM (designated MHM-#, where # indicates pH) was used to test the effect of pH on growth rate, amended with 10 mM glucose (strain KBS 83) or 0.3× R2B (above) (strain KBS 96). Morpholineethanesulfonic acid (MES) was used to buffer at pH ranges between 5.0 to 6.0, 4-morpholinepropanesulfonic acid (MOPS) was used at pH ranges between 6.5 to 7.0, and citric acid was used as a buffer below pH 5.0. All buffers were used at a final concentration of 20 mM. Culture tubes (Bellco catalog no. 2048-18150) containing 5 ml of medium were stoppered under an air atmosphere and held on a reciprocating shaker, horizontally, operating at ca. 50 strokes/min to disrupt cellular flocs. Optical density at 600 nm was monitored periodically with a Thermo Spectronic model 20D+ spectrophotometer. The inoculum consisted of a 1:100 dilution of cultures in mid- to late-log-phase growth (ca. 1×10^8 cells/ml) in MHM-5 with 10 mM glucose (strain KBS 83) or MHM-5 with 0.3× R2B (strain KBS 96).

(iii) Capacity to utilize nitrate. Strains KBS 83 and KBS 96 were tested for their ability to grow anaerobically and reduce nitrate by determining the concentration of ammonium, nitrite, nitrous oxide and nitrate at the beginning and end of incubation. Strains were grown in MHM-5.5 with 10 mM glucose with a headspace of helium in stoppered culture tubes (Bellco catalog no. 2048-18150) at room temperature. Nitrate, nitrite, and ammonium were measured at the Michigan State University Soil Testing Laboratory using the Lachat QuickChem automated flow injection ion analyzer. Nitrous oxide and carbon dioxide were measured using a Shimadzu thermal conductivity gas chromatograph series GC-2014 with the following settings: flow rate, 60 ml/min; column temperature, 55°C; injector temperature, 100°C; detector temperature, 100°C; thermal conductivity, 175 mA. Glucose concentrations were determined using the glucose assay kit (Sigma-Aldrich Co., St. Louis, MO).

(iv) Carbon utilization. Strains KBS 83 and KBS 96 were tested for their ability to oxidize various carbon sources. Strains were grown under air on the following carbon sources for ca. 30 days at room temperature, with a 10 mM final concentration in MHM-5.5: D-glucose, D-fructose, D-galactose, D-mannose, D-ribose, D-xylose, L-arabinose, D-mannitol, D-sorbitol, sucrose, D-maltose, D-raffinose, succinate, acetate, formate, pyruvate, benzoate, ferulate, resorcinol, syringate, and trimethoxybenzoate. Methyl cellulose, pectin, cellulose, and a xylan mix, containing xylan from oat spelts, birchwood, and larch, were used with MHM-5.5 at a final concentration of 0.1% (wt/vol). Due to an unknown auxotrophic requirement, a small amount of yeast extract (5 μg/ml) was added to the medium for growth of strain KBS 96; growth of each respective carbon source was determined after the amount of growth on 5 μg/ml yeast extract alone was subtracted. Growth was determined by visual inspection and/or optical density measurements using a Thermo Spectronic model 20D+ spectrophotometer at an optical density of 600 nm.

(v) Oxygen studies. Strain KBS 83 was tested for its ability to grow under hypoxic conditions with the following concentrations of oxygen in the headspace (vol/vol oxygen, balanced with He): 2%, 4%, 8%, 16%, and 21% on MHM-5.5. Sealed sidearm flasks (500 ml each) along with a low final concentration of 2.5 mM glucose were used to ensure that the targeted oxygen concentration was not depleted throughout these growth experiments. Similar experiments were performed for strain KBS 96; however, this strain was grown on MHM-5 with a mixture of organic compounds (yeast extract, protease peptone, Casamino Acids, and glucose, 0.05 g/liter each) due to an unknown auxotrophic requirement for better growth. The headspace was purged every third day of growth, and oxygen was added again at the appropriate concentration. Strains were grown at room temperature, on an orbital shaker at 150 rpm, and growth was monitored using a Thermo Spectronic model 20D+ spectrophotometer at an optical density of 600 nm. The inoculum consisted of a 1:100 dilution of cultures in late-log-/early-stationary-phase growth (ca. 1×10^8 cells/ml) in MHM-5 with the respective carbon source, presumed to be depleted in oxygen.

(vi) Other physiological tests. Catalase and oxidase tests were performed using standard methods (63, 66). *Escherichia coli* strain REL 607, a derivative of *E. coli* B/r (39), was used as a positive control for the catalase test and as a negative control for the oxidase test. *Pseudomonas aeruginosa* ATCC 10145 was used as a positive control for the oxidase test.

(vii) Colony and cell morphology. Bacterial colonies on plates of MHM medium were examined with a Nikon SMZ-2T dissecting microscope at ×10 to ×15 magnification for size, pigmentation, form, elevation, and margin (66). Gram stain reactions were performed as described previously (63), and cells were stained with India ink to test for bacterial capsules. Gross cell morphology and motility were assessed by using phase-contrast microscopy with a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY). Transmission electron microscopy (TEM) was completed at the Michigan State University Center for Advanced Microscopy (www.ceo.msu.edu).

(viii) Characterization of genomic DNA. The moles percent G+C content of genomic DNA from strains KBS 83 and KBS 96 was determined as described previously (45). Briefly, genomic DNA was extracted using a Qiagen genomic DNA extraction kit (Qiagen, Valencia, CA), and approximately 2 μg of DNA was digested with P1 nuclease and alkaline phosphatase. The nucleosides were separated and quantified using a Shimadzu high-pressure liquid chromatograph fitted with a UV detector and a VP Series Alltima C_{18} column (250 by 4.6 mm; particle size, 5 μm) (Alltech Associates, Inc., Deerfield, IL). Genomic DNA purified from *A. capsulatum* (ATCC 51196) was used as a positive control.

The number of 16S rRNA-encoding genes was determined by nonradioactive Southern hybridization after restriction endonuclease digestion of genomic DNA, as described previously (27, 28). A digoxigenin (DIG)-labeled, *Acidobacteria*-targeting 16S rRNA gene probe was made targeting the region between 31 and 531 (*E. coli* numbering). Genomic DNA purified from *A. capsulatum* was used as a positive control. The number of the *rrs* gene copies for strains KBS 83 and KBS 96 along with the *Terriglobus* strains (15) can be found at the *rrn*DB (http://ribosome.mmg.msu.edu/rrndb/index.php) (38).

(ix) 16S rRNA gene phylogeny of new strains. A nearly full-length sequence of the 16S rRNA gene (ca. 1,400 nucleotides) was generated. Briefly, the 16S rRNA gene was amplified using the broadly inclusive bacterial forward primer 8F and bacterial reverse primer 1492R (34) with the PCR conditions described above. PCR products were cloned with the Invitrogen TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA), reamplified with the modified M13 primers, and purified with ExoSAP-IT (USB, Cleveland, OH). Purified PCR products were submitted for sequencing with 31F, 1492R, 338F (5′ ACT CCT ACG GGA GGC AGC 3′), 338R (5′ GCT GCC TCC CGT AGG AGT 3′), 531R (5′ TAC CGC GGC TGC TGG CAC 3′), 810R (5′ GGC GTG GAC TTC CAG GGT ATC T 3′), 776F (5′ AGC AAA CAG GAT TAG ATA CCC TGG 3′), 1087F (5′ GGT TAA GTC CCG CAA CGA 3′), modified-M13F (F2) (5′ CAG TCA CGA CGT TGT AAA ACG ACG GC 3′), and modifed-M13R (F4) (5′ CAG GAA ACA GCT ATG ACC ATG 3′) (25). Each primer was used in duplicate sequencing reactions. Sequencing was performed at the Michigan State University Research Technology Support Facility (http://rtsf.msu.edu/).

Data analysis. (i) Comparison of acidobacterial 16S rRNA gene sequences from soil surveys. The high-quality, nearly full-length (ca. 1,400-bp) acidobacterial 16S rRNA gene sequences for each soil type were analyzed for differences at the subdivision level. When characterizing these soils at the subdivision level, 100 clones per sample is more than sufficient when looking for differences (ca. 85% operational taxonomic unit [OTU] definition) based on rarefaction analysis (see section S1 in the supplemental material).

Sequences were base called, assembled, and trimmed using the Fincon program (LANL JGI internal program, courtesy of Cliff Han). Sequences were initially classified into the acidobacterial subdivisions using the Ribosomal Database Project Release 10 classifier (8). To confirm these classifications, sequences were aligned with the SILVA database (50), manually checked, and analyzed, and subdivision clusters were determined using well-established reference sequences (4, 21) for generating neighbor-joining phylogenetic trees with ARB software (41). The structures of the agricultural and managed grassland soil acidobacterial communities were compared using *J*-LIBSHUFF (62). When exploring correlations between the relative portions of the acidobacterial subdivisions to edaphic properties (such as soil pH, carbon, and nitrogen), these acidobacterial 16S rRNA gene clone libraries, along with seven smaller ($n = 50$), unreplicated libraries from various treatments at the KBS LTER, were used in

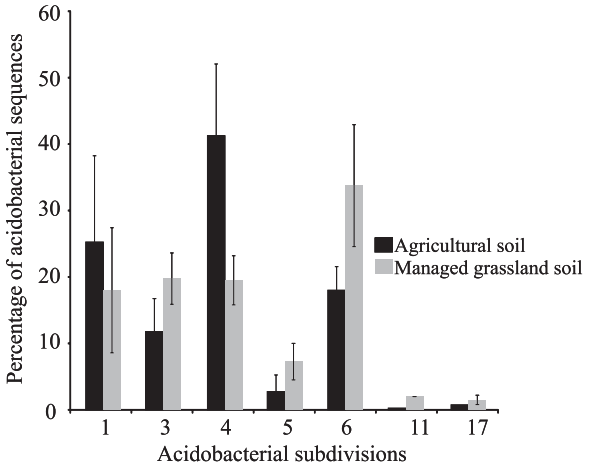

FIG. 1. Structure of acidobacterial communities in agricultural and managed grassland soils in MI based on acidobacterial 16S rRNA gene surveys. Average proportion (± standard deviation) of acidobacterial subdivisions in the agricultural and managed grassland soils at the KBS LTER from four field replicates.

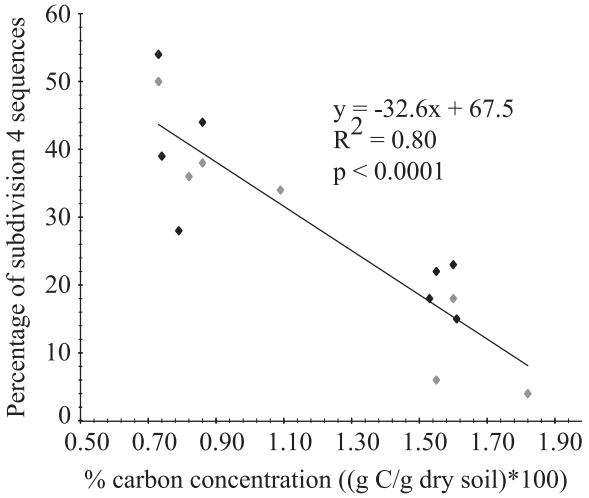

FIG. 2. Relationship between the average percentage of acidobacterial subdivision 4 sequences and carbon concentration for the upper 7 centimeters of soil from the agricultural and managed grassland soils. The four field replicates for each soil treatment are depicted as black boxes, whereas additional unreplicated acidobacterial libraries included in this analysis are depicted as gray boxes. These libraries were generated from other treatments at the KBS LTER. Carbon data were averaged for each treatment from historical data obtained from the KBS LTER website for carbon data collected between 1989 and 2001. Raw data and a detailed description of the treatments can be found at the KBS LTER website (http://lter.kbs.msu.edu/).

the analysis. These libraries were created and analyzed using the same methods described above.

(ii) Comparison of acidobacterial 16S rRNA gene sequence enriched on PP, ROC, and xylan. Partial sequences (ca. 400 bp) of acidobacteria detected on enrichment plates were analyzed for (i) subdivision-level placement using a maximum likelihood algorithm (RAxML) in ARB, (ii) differences in genetic distance, and (iii) community structure between the carbon sources. The maximum likelihood phylogenetic tree utilized a filter (*E. coli* positions 53 to 396) to ensure comparison of the same regions for these partial sequences. RAxML was used for bootstrapping analysis (69). The genetic distance of the acidobacterial 16S rRNA gene sequences recovered from these and previous plates (15, 70) was determined using MEGA4 (72). Differences in the acidobacterial communities that arose on readily oxidizable and plant polymers were assessed for differences using ∫-LIBSHUFF (62) and Unifrac (40).

(iii) Analysis of nearly full-length 16S rRNA gene sequence of new strains. Sequences were assembled using the DNA Star LaserGene software (Madison, WI), aligned using the ARB software (41), and compared to acidobacterial 16S rRNA gene sequences downloaded from SILVA (50). The maximum likelihood algorithm (AxML) in ARB was used for the generation of the phylogenetic trees with a terminus filter to ensure comparison of the same regions (*E. coli* positions 32 to 1461). PAUP* version 4.0b10 was used for bootstrapping analysis (71).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of the acidobacterial strains, KBS 83 and KBS 96, were deposited in GenBank with accession numbers FJ870383 and FJ870384, respectively. The acidobacterial 16S rRNA gene sequences from the soil survey (HM061696 to HM062495) and the acidobacterial plate wash PCR sequences (HQ229956 to HQ229988, FJ870379 to FJ870384) have also been deposited in GenBank. Additional sequences used in the acidobacterial plate wash PCR sequence analysis were previously submitted (DQ660892 to DQ660895, AY587227).

RESULTS AND DISCUSSION

Comparison of acidobacterial subdivisions present in the agricultural and managed grassland soils. Plant litter and root exudates are abundant sources of carbon and energy for microbial communities in fertile soils. To better define the potential niches of acidobacteria in soil, in particular their potential role in degrading plant biomass, we conducted a DNA-based survey of the distribution of acidobacteria in agricultural and managed grassland soils, compared the composition of acidobacteria isolated from these soils with growth media amended with plant polymers or readily oxidizable carbon, enriched for isolates using plant polymers, and characterized selected strains that were capable of using plant polymers as well as simple sugars for growth.

The specific phylogenetic affiliations of nearly full-length acidobacterial 16S rRNA gene sequences from the agricultural and managed grassland soils revealed large proportions of subdivisions 1, 3, 4, 5, and 6 and minor contributions of subdivisions 11 and 17 (less than 2% of the acidobacterial community) (Fig. 1). Only acidobacterial 16S rRNA gene sequences were recovered. Members of the remaining subdivisions were not detected, possibly due to mismatches between the forward primer and the 16S rRNA genes from these subdivisions, as noted previously when the *Acidobacteria*-targeting 31F primer was designed (5), or because they were not abundant enough to be detected in our clone libraries.

The compositions of the acidobacterial communities differed significantly between these soils based on ∫-LIBSHUFF analysis ($P < 0.0001$) (62). The agricultural soils were dominated by subdivision 4 sequences that comprised 41% of the total acidobacterial clone library, while the managed grassland soil contained only 19% subdivision 4 sequences. The increase in subdivision 4 sequences in the agricultural soil relative to the managed grassland soil was not due to the increased abundance of a particular sequence, and the overall richness of this group increased, as illustrated by the appearance of new clades (see section S2, gray boxes of subdivision 4, in the supplemental material). Sequences from the managed grassland soil were more evenly distributed among the dominant subdivisions (1, 3, 4, and 6), with an average percentage across these subdivisions of ca. 23% (Fig. 1).

Agricultural and managed grassland soils were characterized

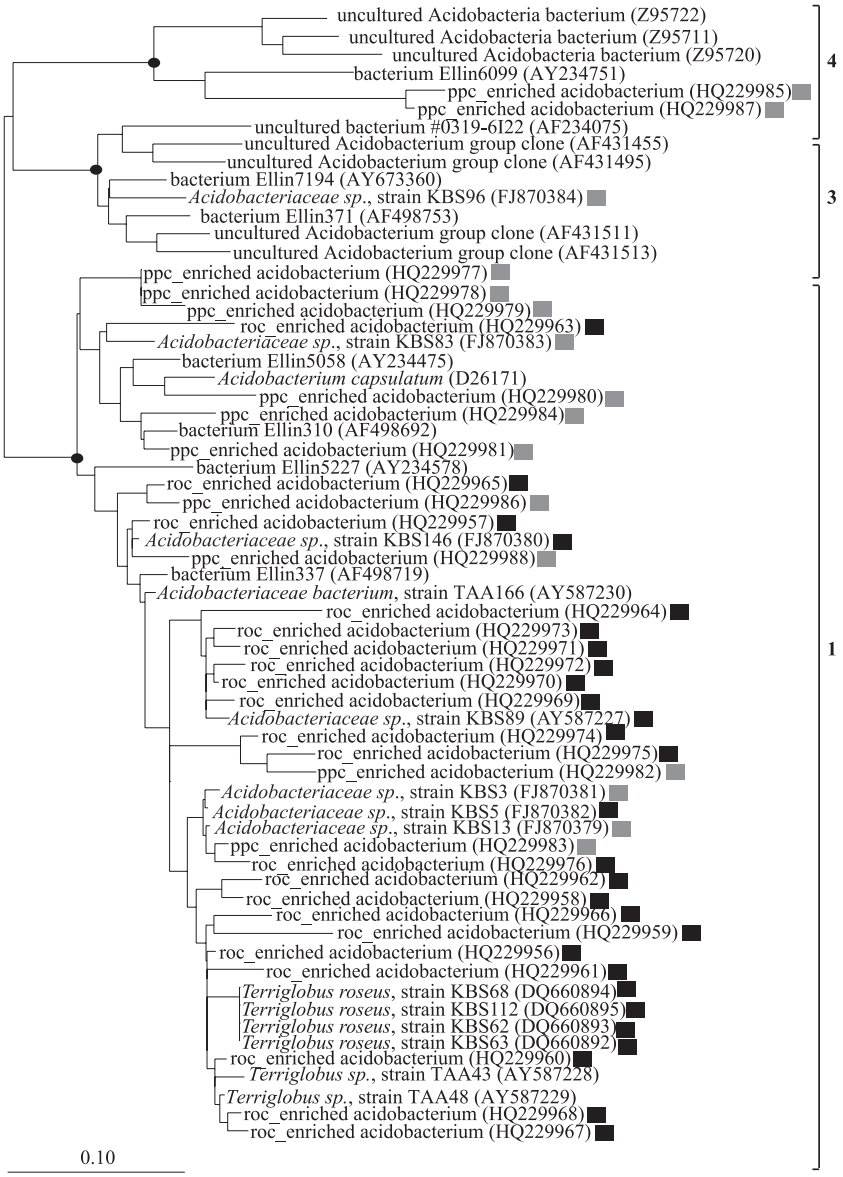

FIG. 3. Summary of acidobacterial sequence diversity. Maximum likelihood tree based on 16S rRNA gene sequences from cultivated representatives, environmental clones, and partial acidobacterial sequences detected from cultivation experiments using readily oxidizable carbon (black box, $n = 28$) and plant polymers (gray box, $n = 16$). Subdivision nodes supported by a bootstrap value of 100% are indicated with a filled circle (●). *Geothrix fermentans* and *Holophaga foetida* of subdivision 8 were used as an outgroup (not shown). The scale bar indicates 0.10 changes per nucleotide.

based on pH, moisture, and percentage of carbon and nitrogen (g C or N per 100 g soil) (Table 1). The managed grassland soil had ca. 2-fold-higher levels of soil moisture, carbon, and nitrogen. To explore potential drivers of the dominance of subdivision 4 sequences in the agricultural soil, we examined correlations between the percentages of subdivision 4 sequences with various edaphic properties, such as moisture, pH, nitrogen, and carbon concentrations. Carbon availability appeared to be one of the factors influencing the composition of the acidobacterial community. There was a significant, negative correlation between the portion of subdivision 4 sequences and the organic carbon concentration ($P < 0.0001$; Fig. 2). This observation is consistent with the finding that subdivision 4 acidobacteria are more abundant in arid soils which are also low in total carbon (C. R. Kuske, unpublished data) and the recent finding that a putative subdivision 4 acidobacterium harbors pathways for the fixation of carbon dioxide (7).

Cultivation of aerobic heterotrophs using readily oxidizable carbon and plant polymers. In an effort to isolate new acidobacterial strains, agricultural and managed grassland soils were used as inocula for media containing either plant polymers or readily oxidizable carbon as the primary carbon and energy source. The plant polymer medium contained compounds representing some of the major constituents of plant biomass: methyl cellulose, xylan (derived from oat spelts, larch, and birchwood), and pectin, along with gellan gum as the

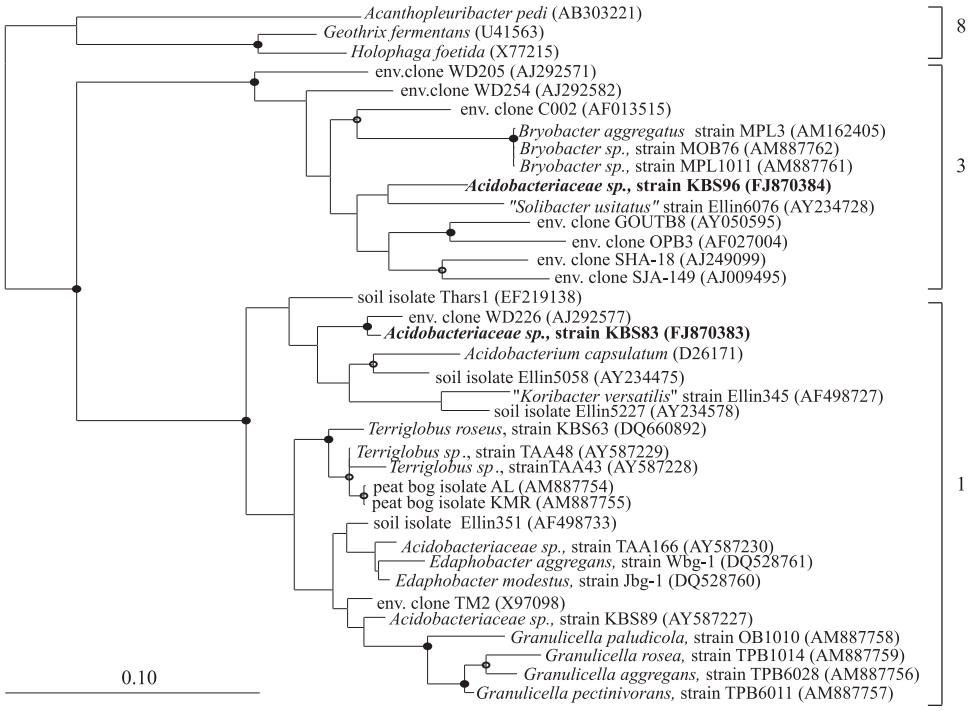

FIG. 4. Maximum likelihood tree of the acidobacterial subdivisions 1, 3, and 8 (indicated to the right of the group) based on 16S rRNA genes using sequences obtained from cultivated representatives and environmental clones. *Geothrix fermentans*, *Holophaga foetida*, and *Acanthopleuribacter pedi* of subdivision 8 were used as an outgroup. Strains from this study are in bold font. Internal nodes supported by a bootstrap value of >95% are indicated with a filled circle (●) and those of >70% with an open circle (○). The scale bar indicates 0.10 changes per nucleotide.

solidifying agent. The inclusion of plant polymers reduced the total recovery of bacteria from both agricultural and managed soils by approximately 6-fold compared to the medium containing readily oxidizable carbon (ca. 3.5×10^7 [plant polymers] versus ca. 0.6×10^7 [readily oxidizable carbon] CFU/gram of soil [dry weight]).

These enrichment plates were screened for the presence of acidobacterial colonies using plate wash PCR with *Acidobacteria*-targeting primers. A total of 44 acidobacterial 16S rRNA gene sequences were obtained from plates amended with plant polymers ($n = 16$) and readily oxidizable carbon ($n = 28$; this study and previous studies [15, 70]). The acidobacterial sequences were aligned and analyzed for subdivision-level placement. The null hypothesis was that the subdivision-level placements of acidobacteria growing on readily oxidizable carbon and plant polymers were equal given the similar enrichment methodologies.

Plates amended with plant polymers resulted in the growth of more phylogenetically diverse acidobacteria than the readily oxidizable carbon plates. Members of subdivisions 1, 3, and 4 were identified on plates amended with plant polymers (Fig. 3, gray boxes), while only subdivision 1 sequences were identified on plates amended with readily oxidizable carbon (Fig. 3, black boxes). The average genetic distance among acidobacterial sequences detected when plant polymers were used as the carbon source was ca. 12%, compared to ca. 2% on plates amended with readily oxidizable carbon based on MEGA4 (72). The phylogenetic composition of acidobacteria enriched with plant polymers was significantly different from the composition that appeared on plates with readily oxidizable carbon (Unifrac $P < 0.01$; $\int$-LIBSHUFF $P < 0.05$). The increased genetic distance of acidobacterial sequences from plates amended with plant polymers suggests that plant polymers select for more diverse acidobacteria than acidobacteria cultivated with readily oxidizable carbon.

The inocula of these plates came from either agricultural or managed grassland soil management regimes. The patterns of carbon use by acidobacteria were further compared between the two soil management regimes. A significant difference ($P < 0.03$, Unifrac significance test) between the managed grassland and agricultural soils was identified, indicating that the acidobacteria capable of plant polymer degradation were not the same in the two soil regimes. The distribution of this carbon utilization phenotype clearly warrants further study of these and other soils.

Characterization of novel plant polymer-degrading acidobacterial strains. Six new acidobacterial strains were isolated during these cultivation studies. Two novel strains, KBS 83 and KBS 96, were further characterized. Both strains were isolated from VL-5.5-PP from the agricultural soil after incubation at room temperature for ca. 30 days in air. Figure 4 depicts a phylogenetic tree of nearly full-length sequences for the new strains. Strains KBS 83 and 96 were compared to closely related, validated genera such as *Acidobacterium* (26), *Terriglobus* (15), *Edaphobacter* (29), *Bryobacter* (31), and *Granulicella* (47). Strain KBS 83 is a member of subdivision 1 and is distantly related to the genera *Acidobacterium*, *Terriglobus*, *Edaphobacter*, and *Granulicella*, with sequence identities of ca. 94%, 92%, 94%, and 91%, respectively. It is most similar (ca. 98%) to an environmental clone from

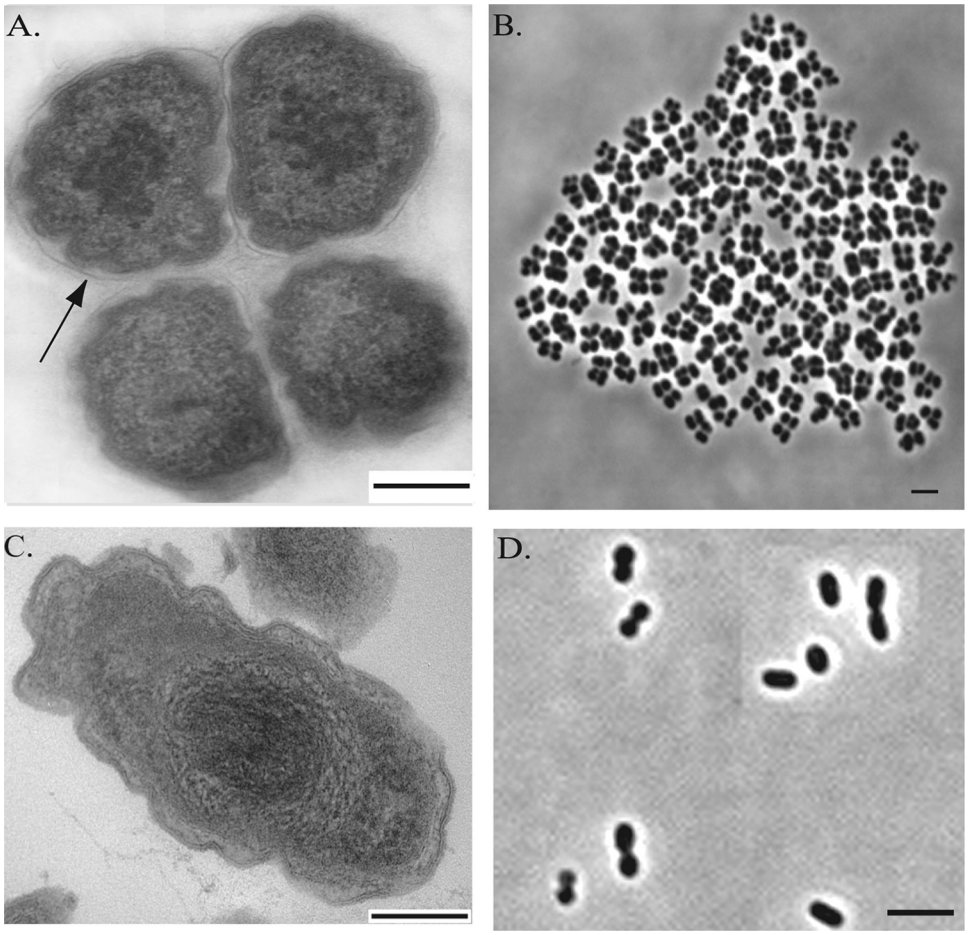

FIG. 5. Phase-contrast and transmission electron micrograph of KBS 83 (A and B) and KBS 96 (C and D). The arrow in panel A indicates the capsular material layer produced by KBS 83. Scale bars indicate 1 μm (B and D) and 200 nm (A and C).

a polychlorinated biphenyl-polluted soil (clone WD226, AJ292577) (46). Strain KBS 96 is a member of subdivision 3 and is ca. 91% identical to strains in the genus *Bryobacter* and ca. 93% identical to "*Candidatus* Solibacter usitatus" strain Ellin 6076.

Colonies of the newly described acidobacteria were small, approximately 1 mm in diameter (after ca. 14 to 21 days of incubation), and had a circular form with a convex elevation and undulate margin when grown on MHM-5 with glucose under air. Colonies of strain KBS 83 were smooth, glutinous white colonies, whereas the colonies of strain KBS 96 had smooth, butyrous pale-yellow-pigmented colonies. These strain took approximately 2 to 3 weeks to form a visible colony on the surface of the agar plate. Neither strain KBS 83 nor KBS 96 was pigmented like strains of the *Terriglobus* genus (15).

Cells of both strains were short, plump, Gram-negative, nonmotile rods measuring ca. 0.5 μm by 0.4 μm (strain KBS 83) and 1 μm by 0.4 μm (strain KBS 96) when grown on MHM-5 with glucose. Cells possessed exaggerated convoluted outer membranes when viewed by TEM, as is typical of a Gram-negative type cell wall (Fig. 5A and B [strain KBS 83] and C and D [strain KBS 96]). Both strains produced an extracellular matrix of as-yet-unknown chemical composition, which caused cells to stick together tightly in colonies and form visible clumps in liquid culture similarly to the previously isolated strains in the *Terriglobus* genus (15). Strain KBS 83 typically appeared in clumps of four cells, as seen under phase-contrast and transmission electron microscopy (Fig. 5A and B), and was held together in this tetrad by capsular material visible in electron micrographs (Fig. 5A). Capsules or extracellular polysaccharides have been shown to promote bacterial adhesion (9) and soil aggregation (1, 3) and prevent desiccation (52), all of which would be beneficial for a soil microorganism.

The characteristics of the new strains are summarized in Table 2. Strains KBS 83 and KBS 96 preferred mildly acidic pH conditions. Growth was observed for strain KBS 83 over a pH range of 4.5 to 6.0 (growth rates were highest at 5.0) and for strain KBS 96 over a pH range of 4.0 to 6.0 (growth rates were highest at 6.0). The preference of these strains for mildly acidic pH is consistent with the pH of their native soil environment.

Strains KBS 83 and KBS 96 were tested for their ability to use nitrate via either denitrification, nitrate reduction to ammonium, or nitrate reduction. Under the conditions tested, neither strain appeared capable of denitrification or dissimilatory nitrate reduction to ammonia. Strain KBS 96 reduced a small percentage (ca. 3%) of nitrate to nitrite.

The carbon utilization profiles of these new strains were compared to those of previously characterized strains, specif-

TABLE 2. Phenotypic characteristics of *Terriglobus*, *Acidobacteriaceae*, *Granulicella*, *Bryobacter*, and *Edaphobacter* strains[a]

Characteristic	Result for strain or group						
	Acidobacteriaceae KBS 83	*Terriglobus* KBS 63[b]	*Edaphobacter*[c]		*Granulicella* (summary of genus results)[d]	*Acidobacteriaceae* KBS 96	*Bryobacter* MPL3T[e]
			Jbg-1	Wbg-1			
Subdivision	1	1	1	1	1	3	3
Origin	Agricultural soil	Successional community soil	Alpine soil	Forest soil	*Sphagnum* peat and *Cladonia* sp.	Agricultural soil	Acidic *Sphagnum* peat
Gram reaction	Negative	Negative	Negative	Negative	−	Negative	Negative
Cell shape	Rod	Rod	Short ovoid rods	Short ovoid rods	Rod	Rod	Coccoid or short rods
Length (μm)	ca. 0.5	1.1 ± 0.2	ca. 1.4	ca. 1.8	ca. 1.5–15	ca. 1	ca. 0.5–0.8
Width (μm)	ca. 0.4	0.6 ± 0.1	ca. 0.6	ca. 0.6	ca. 0.4–1.0	ca. 0.4	ca. 0.8–1.1
Capsule	+	−	−	−	ND	−	+
Pigment	White	Pink	−	−	Pink or red	Pale yellow	−
rrs copy no.	1	2	ND	ND	ND	2	ND
G+C content (mol%)	62.2 ± 0.07	59.8 ± 0.5	55.8	56.9	57.3–59.3	60.6 ± 0.41	55.6
Catalase	+	+	+	+	+	+	+
Oxidase	−	−	+	−		+	−
Motility	−	−	+	−	−	−	−
pH range (optimum)	4.5–6.0 (5)	5.0–7.0 (6)	4.5–7.0 (5.5)	4.0–7.0 (5.5)	3.0–7.5 (3.8–4.5)	4.0–6.0 (6)	4.5–7.2 (5.5–6.5)
Growth at:							
4°C	−	−	ND	ND	+	−	+
12°C	+	+	ND	ND	+	+	+
25°C	+	+	+	+	+	+	+
37°C	+	−	−	+	−	+	−
Degradation of select complex carbon							
Xylan	+	+	+[d]	+[d]	+	+	−
Cellulose	+	ND	ND	ND	−	+	−
Pectin	+	−	−	−	+	+	+
Methyl cellulose[f]	+	−	ND	ND	−	+	−
Starch	ND	+[d]	+[d]	+[d]	+	ND	+

[a] Strains KBS 83 and KBS 96 utilized D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, L-arabinose, D-sorbitol, sucrose, D-maltose, D-raffinose, methyl cellulose, ferulate, and syringate but did not utilize sodium acetate, sodium pyruvate, sodium formate, benzoate, and resorcinol. Only strain KBS 96 utilized D-ribose and D-mannitol. Only strain KBS 83 utilized sodium succinate and trimethylbenzoate. ND, not determined.
[b] Reference: characteristics described in a previous study (15).
[c] Reference: characteristics described in a previous study (29).
[d] Reference: characteristics described in a previous study (47).
[e] Reference: characteristics described in a previous study (31).
[f] Carboxymethylcellulose was tested for strains of the genera *Granulicella* and *Bryobacter*.

ically *Edaphobacter modestus* strain Jbg-1, *Edaphobacter aggregans* strain Wbg-1 (29), strains in the genus *Granulicella* (47), *Bryobacter aggregatus* (31), and *Terriglobus roseus* strain KBS 63 (15). The metabolic characteristics of these strains are summarized in Table 2. The newly isolated strains have a broader metabolic capacity which distinguishes them from previous acidobacterial strains (15) and extends the potential niches that might be occupied by acidobacteria to the degradation of plant polymers in soil. Two new strains capable of plant polymer degradation, KBS 83 and KBS 96, were capable of growth on a diverse collection of complex organic compounds, including xylan, cellulose, methyl cellulose, syringate, pectin, and ferulate (Table 2). Like previous strains, KBS 83 and KBS 96 were also able to grow on a range of mono-, di-, and trisaccharides that are the primary sugars in plant root exudate: glucose, fructose, sucrose, maltose, galactose, xylose, arabinose, and raffinose (42). In comparing the carbon utilization profiles of our new strains with those of other aforementioned strains (Table 2), it appears that members of subdivision 1 and 3 are able to utilize many forms of plant polymers.

The versatility of heterotrophic metabolism in the phylum *Acidobacteria* has also been documented in the genomes of three previous characterized strains with the potential to oxidize a range of carbon substrates, including chitin, starch, xylan, and pectin (75). In addition, these genomes are populated with various oxygenases presumably used in the degradation of aromatic compounds.

Members of the phylum *Acidobacteria* have been found both in bulk soil (5, 22) and in the rhizosphere (32, 37). It is reasonable to suggest that their versatility in heterotrophic metabolism allows them to exploit various niches in the soil environment, for example, plant polymer degradation in bulk soil and readily oxidizable carbon in the rhizosphere. Although acidobacteria may not grow quickly, their genomes encode high-affinity ABC transporters for sugars (75) that could provide a competitive advantage in the rhizosphere when carbon concentrations are low. Furthermore, these newly isolated strains contain either one (strain KBS 83) or two (strain KBS 96) copies of the *rrs* gene (Table 2), a genomic marker for oligotrophy in bacteria (27). Oligotrophic bacteria, such as acidobacteria, have the capacity to survive in environments where nutrients are low (16). Taken together, these data suggest that select members of subdivision 1 and 3 in the phylum *Acidobacteria* have the potential to play an active role in the degradation of plant polymers in bulk soil and utilize sugars

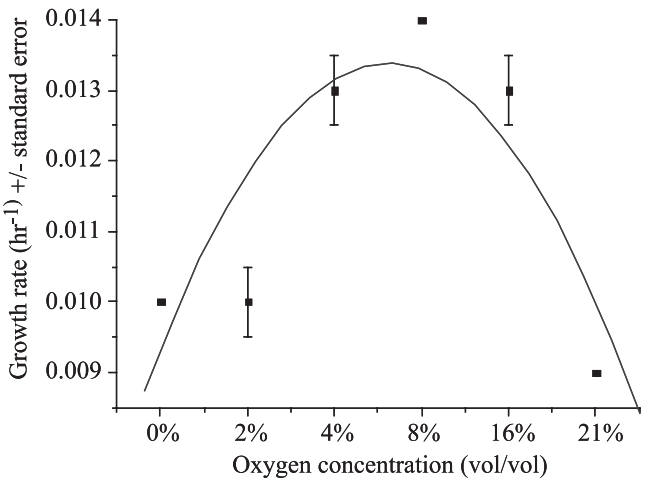

FIG. 6. Summary of growth rates for strain KBS 83 under different concentrations of oxygen (vol/vol oxygen balanced with He). Each black box represents the average growth rate ± standard error at the respective oxygen concentration.

from plant root exudates at various concentrations in the rhizosphere.

The heterogeneity of soil creates not only habitats with different types and concentrations of carbon but also gradients of oxygen. Oxygen can be depleted as soil moisture increases (6, 48, 74), creating transition zones between oxic and anoxic conditions (20, 64). These transition zones provide a habitat for microaerophiles, organisms not capable of growing or growing poorly under atmospheric concentrations of oxygen, presumably due to an increased sensitivity to toxic forms of oxygen such as H_2O_2, O_2^-, and OH· (30).

Strain KBS 83 grew significantly faster at oxygen concentrations between 4% and 16% (avg. P value = 0.01) than at atmospheric concentrations of oxygen (21%, vol/vol); it grew optimally at 8% oxygen ($\mu = 0.014\ h^{-1}$) (Fig. 6), indicating that it is microaerophilic. Strain KBS 96 grew qualitatively better under atmospheric concentrations of oxygen. Although strain KBS 83 was catalase positive, illustrating that cells have the capacity to protect against peroxides, this enzyme's effectiveness might be low, similar to that of certain *Campylobacter* species classically described as microaerophiles (55). Previous work demonstrated that the inclusion of catalase, as well as incubation under microoxic conditions, increased the frequency of acidobacterial detection by ca. 3.3- and 1.3-fold, respectively, in initial cultivation experiments (15, 70). Members of the genus *Terriglobus* produce a carotenoid(s) which is differentially synthesized in response to oxygen concentrations, suggestive of an oxidative stress response (15).

In summary, the influence of organic resources was assessed to explore the distribution and cultivation of acidobacteria in agricultural and managed grassland soils in Michigan. The inclusion of plant polymers in the enrichment medium increased the diversity of acidobacteria cultivated but decreased the total number of heterotrophs recovered compared to the results with readily oxidizable carbon. Two novel plant polymer-degrading acidobacteria were isolated and characterized. The capacity to degrade plant polymers, along with other characteristics described in this report, helps define niches of these slow-growing bacteria.

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